

Atherosclerosis Development and the Inflammatory Response of Hepatocytes to Sesame Oil
Supplementation

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Abstract

Atherosclerosis is one of the leading causes of death. Preventing atherosclerosis starts with controlling the build-up of plaque in the arteries. Due to the current obesity epidemic, preventing atherosclerosis through the diet is increasingly important. The types of dietary fats consumed may lead to changes in plaque content and reduce risk for plaques to form lesions that stick to the arterial wall. Dietary sesame oil reduces arteriosclerotic plaque formation in mice prone to developing atherosclerosis. Because the liver is the major site for cholesterol metabolism and therefore, contributes to atherosclerotic disease in this way, we hypothesize a sesame oil diet would reduce inflammation in the livers of mice. Using the LDLr ^{-/-} mouse, which is prone to atherosclerosis, we measured mRNA for several genes coding for inflammation and infiltration of macrophages (e.g., IL-6, TNF α , TNF α receptor, and F4/80) in the liver. In contrast to our hypothesis, markers of inflammation appear to be elevated in mice fed sesame oil. We suspect the elevation of the inflammation markers measured was an indirect effect of dietary sesame oil inducing lipid accumulation in the liver. In order to test the causative effect of sesame oil on lipid accumulation and inflammation, we will quantify the fat content (triglyceride, diglyceride and monoglyceride) in the liver of mice fed the sesame oil. Once we have a better assessment of dietary induced changes of the liver lipid composition, we will know more about why livers are exhibiting enhanced markers of inflammation in mice fed sesame oil diets.

Introduction

Atherosclerosis is a major contributor to heart disease, which has been the leading cause of death in the U.S. in recent years (1). It is believed the development of arteriosclerotic plaque is caused by inflammation in the arteries that starts out as a fatty streak on the wall of the artery eventually progressing into a plaque. This plaque build-up becomes dangerous when there is possibility of it rupturing. What determines whether or not a plaque will be prone to rupture is dependent on its makeup (2). Increased levels of cholesterol (3) and saturated fatty acids combined with macrophages and other inflammatory markers may contribute to this potentially life-threatening condition by creating an unstable plaque that may break through the fibrous cap that has formed (4). Vulnerable plaques that rupture through their fibrous cap can be severe enough to cause a blood clot resulting in a heart attack or stroke if the plaque is large enough (i.e. containing many lipids, cholesterol, macrophages, and other debris) (5). Many studies have tested this causal relationship between inflammation and atherosclerotic plaque development in the hopes of finding a way to prevent an inflammatory response. Recently it has been found the amount of fat being consumed is proving to be less important than the types of fats being consumed. Unlike saturated fatty acids, unsaturated fatty acids have shown to lower low-density lipoprotein (LDL)-cholesterol levels while increasing HDL-cholesterol levels (6, 7). This anti-atherogenic effect of monounsaturated fatty acids (MUFAs) and polyunsaturated fatty acids (PUFAs) by increasing HDL-cholesterol levels is promising for unsaturated fatty acids in normal subjects. However, MUFAs, when compared to PUFAs have shown to be pro-atherogenic in LDL receptor knockout mice (8). These LDLr ^{-/-} mice are prone to developing atherosclerosis due to their impaired lipid transport abilities (9). PUFAs have shown to reduce atherosclerosis development in LDLr ^{-/-} mice, which has been the focus of some anti-atherogenic studies recently (10). Sesame oil, which is known to have a 1:1 MUFA:PUFA ratio was chosen in this

study due to its high PUFA levels (11). Sesame oil has also been shown to reduce the formation of the arteriosclerotic lesion in LDLr ^{-/-} mice (12). These points taken together show a promising sign for the anti-atherogenic signs of the PUFAs in sesame oil. There has also been preliminary data for a clinical study involving sesame oil that has shown sesame oil reduces pro-inflammatory markers as well as increasing anti-inflammatory markers in adipose tissue (data not published). With this evidence we hypothesized that a sesame oil diet would reduce inflammation in the livers of mice.

Methods

Mice and Diets Male LDLr ^{-/-} mice (n=12) were divided into two groups of six and were fed a control diet or a sesame diet. The control group was given the Teklad 17% AMF (anhydrous milkfat) diet TD.04287 with .2% cholesterol, Harlan Laboratories Inc, Madison, Wisconsin USA. The sesame group was given the Teklad 17% sesame oil diet TD.04288 with .2% cholesterol, Harlan Laboratories Inc, Madison, Wisconsin USA. The remaining content of the diets was identical. The mice were fed the experimental diets for three months; then were sacrificed and livers collected and preserved for future analysis.

mRNA Analysis of Target Genes in Liver qRT-PCR was used to measure the mRNA levels of four pro-inflammatory gene markers: IL-6, TNF α , TNF α R, and F4/80. 50mg of liver was cut and homogenized for 15 seconds in 1.5ml of qiazol. Samples incubated for 5 min at room temperature (RT) and then 300ul of CHCl₃ was added. Samples were incubated again for 3 minutes at RT and spun at 12000g for 15 minutes at 4°C in our microcentrifuge. The top phase created was removed and 750 ul of isopropanol was added. The tube was then gently inverted 3 times and incubated at RT for 10 minutes. After 10 minutes the samples were spun at 12000g for 10 min at 4°C where a small white pellet of RNA was left. The pellet was dried and then added

to 1.5 ml of 75% ethanol. The mixture was vortexed quickly and then spun at 7500g for 5 minutes at 4°C. The ethanol was removed and 40 ul of RNase-free DI H₂O was added to break up the pellet. The mixture was vortexed and then incubated for 10 minutes at 55°C on a hotplate. After the incubation the sample was stored at -80°C. After the RNA extraction was completed the reverse transcription of RNA to cDNA was next. The RT Master Mix Buffer was made, which included: 24 ul of 10x RT Buffer, 9.6 ul of 25x dNTPs, 24 ul of 10x Random Primers, 12 ul of Multiscribe RTase, and 50.4 ul of RNase-free DI H₂O. 10 ul of RT Master Mix Buffer and 10 ul of sample/RNase-free H₂O were added to each PCR tube. The PCR tubes were mixed, vortexed, and then inverted. The PCR tubes were next put in the thermal cycler for 10 minutes at 25°C, 120 minutes at 37°C, 5 minutes at 85°C, then 60 minutes at 4°C. The newly created cDNA was then stored long-term at -25°C. The TaqMan Universal PCR Master Mix reagent with primers was made next, which included: 300 ul of TaqMan Universal PCR Master Mix, 30 ul of RNase-free DI H₂O, and 30 ul of TaqMan gene expression target gene, or 30 ul of TaqMan endogenous control. The target gene and endogenous control were separated so the plates could be run singleplex. ddCt analysis was then used to compare the cycle thresholds of each gene to housekeeping mRNA for GAPDH.

Lipid Concentration of Liver The total fat content in the liver was calculated to compare between the two diet groups. The livers were cut and put into 6 ml of CHCl₃. Before the samples or CHCl₃ were added the test tubes were weighed. 1 ml of 0.88% KCl was added and spun at 1000g for 15 minutes at 4°C. The top 0.88% KCl layer was removed completely and the samples were spun at 1000g for 15 minutes at 4°C. The remaining 0.88% KCl was removed again and the remaining CHCl₃ was dried under the nitrogen dryer. After the CHCl₃ was dried the test tubes were weighed and the difference between the two test tube weights was calculated.

The fat content in the liver was extracted and methylated for fatty acid analysis by gas chromatography (GC). The livers were cut and put in 4 ml CHCl_3 . 1 ml of 0.88% KCl was added and spun at 1000g for 15 minutes at 4°C. The 0.88% KCl layer was removed, 1 ml of CHCl_3 was added, and then respun at 1000g for 15 minutes at 4°C. The remaining 0.88% KCl was removed and the remaining CHCl_3 was dried under the nitrogen dryer. 300 μl of hexane was then added to the tubes, vortexed, and transferred to the GC vials and stored at -25°C. The GC allows for the quantification of various fatty acids present in each sample. The GC quantifies the fatty acids by plotting retention time versus concentration and calculating the percent area that is found under the curve.

Statistical Evaluation of Data delta delta Cycle threshold (ddCt) analysis was used to compare the amplification between the target genes and endogenous control. The delta cycle threshold (dCt) for the control group was calculated by subtracting the average Ct for the endogenous control from the average Ct for the target gene. The average Ct values for the target genes and endogenous control were found next for each mouse whereafter the value for the endogenous control was subtracted from the target gene to calculate the dCt value. The ddCt value was calculated next by subtracting the dCt value found for the entire control group from the dCt value found from each individual mouse. In order to normalize the target gene data to the endogenous control the ddCt values were then taken and put into the equation $2^{(-\text{ddCt})}$ where the values were then averaged for each diet group and their standard deviations calculated. The total lipid content was calculated by subtracting the weight of the empty test tube from the weight of the test tube containing the fat whereafter the average for each diet group was found and the standard deviations calculated. A t test was done to calculate the statistical significance for each value compared to a control value. The p values were considered significant if $p < 0.05$.

Results

The level of F4/80 mRNA in the sesame group was significantly decreased in comparison to the control group (Figure 1). The level of IL-6 mRNA was not significantly different between the sesame group and control group (Figure 2). The mRNA level of TNF α in the sesame group was significantly decreased in comparison to the control group (Figure 3). The level of TNF α R was not significantly different between the sesame group and the control group (Figure 4). The quantity of lipid accumulation in the livers of the control group was significantly higher than amounts of lipid in mice fed control diet. The fatty acid composition of the diet and livers of the mice fed diets are shown in Table 1 (Table 1A, Sesame diet; Table 1B, Control Diet).

Discussion

Our overall hypothesis was that sesame oil would decrease inflammatory markers in the liver. Our finding that the sesame oil diet significantly decreased mRNA levels of F4/80 and TNF α compared to the control fed mice supported our hypothesis. These two transcripts are normally associated with a pro-inflammatory response in the liver, but their decrease in expression of the mice fed the sesame oil diet shows support for our hypothesis of decreasing pro-inflammatory markers in sesame oil fed mice. Sesame oil was shown to decrease total lipids and MUFAs in the liver while increasing PUFAs as well. It is believed PUFAs have the ability to reduce atherosclerosis development, and the high PUFA levels in sesame oil may be related to the significant decrease in mRNA expression of F4/80 and TNF α . MUFAs have also been shown to have pro-atherogenic qualities (8), which could help explain the decrease in the pro-inflammatory F4/80 and TNF α in the sesame oil mice compared to the control group mice. The decrease in the pro-inflammatory gene markers coupled with a decrease in total lipid content in the sesame oil group shows that sesame oil is reducing two major factors for arteriosclerotic

plaque formation. The reduction in the pro-inflammatory markers could have been due to an increase in PUFAs, a decrease in MUFAs, or something else we did not measure, but the signs are promising for sesame oil and specifically the PUFAs in the sesame oil for possibly decreasing an inflammatory response leading to atherosclerotic plaque formation.

Limitations and Future Directions

One limitation is the number of mice used, which were 6 mice per group in this study. Many studies use 15 or more mice per group due to the variability of responses to diet-induced changes in inflammation and responses to the test oil (sesame). A second limitation is that we did not handle the mice; instead, the mice were fed, sacrificed, and their livers by others were sent to us from another lab. Actually seeing the process the entire way from receiving the mice and feeding them, all the way to necropsy would have been advantageous.

Future directions of the study should address other lipid responses in the liver due to supplementing sesame oil. These may include cholesterol metabolism, which was the same in these diets and not measured, but may be able to predict the protective effect of sesame oil for atherosclerosis.

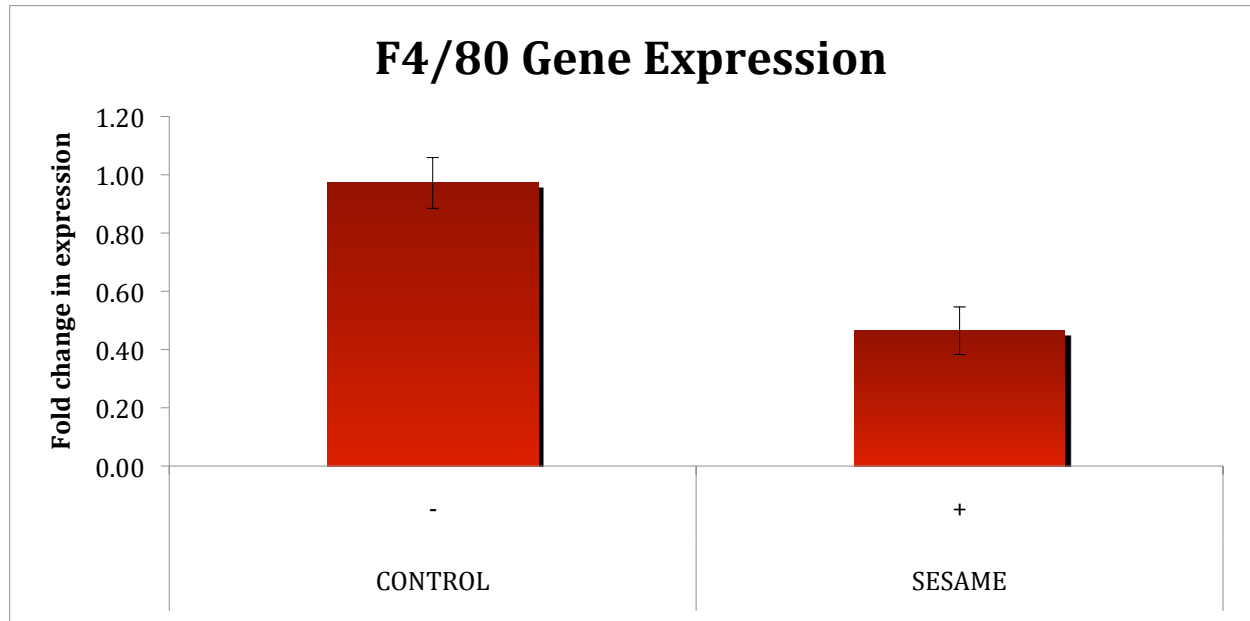


Figure 1: Fold change in expression of F4/80 mRNA levels in the control and sesame oil groups. The sesame oil fed mice had significantly ($p < 0.05$) decreased F4/80 mRNA levels compared to the control group.

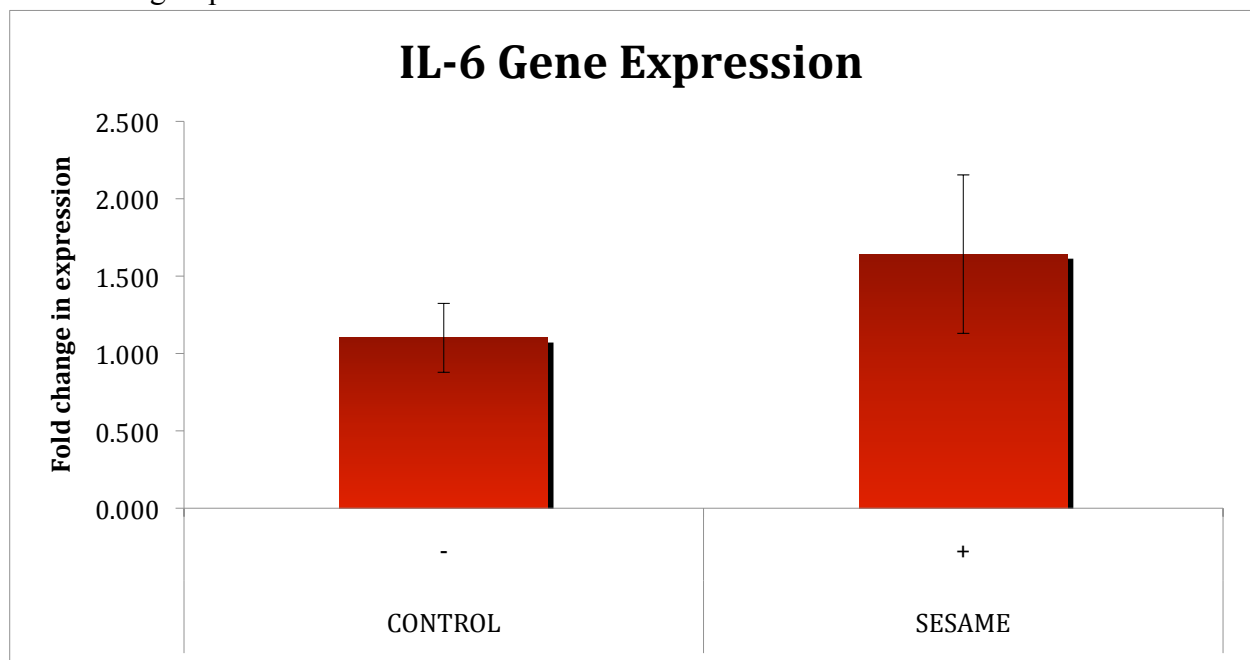


Figure 2: Fold change in expression of IL-6 mRNA levels in the control and sesame oil groups. There was no statistical significance between the two groups.

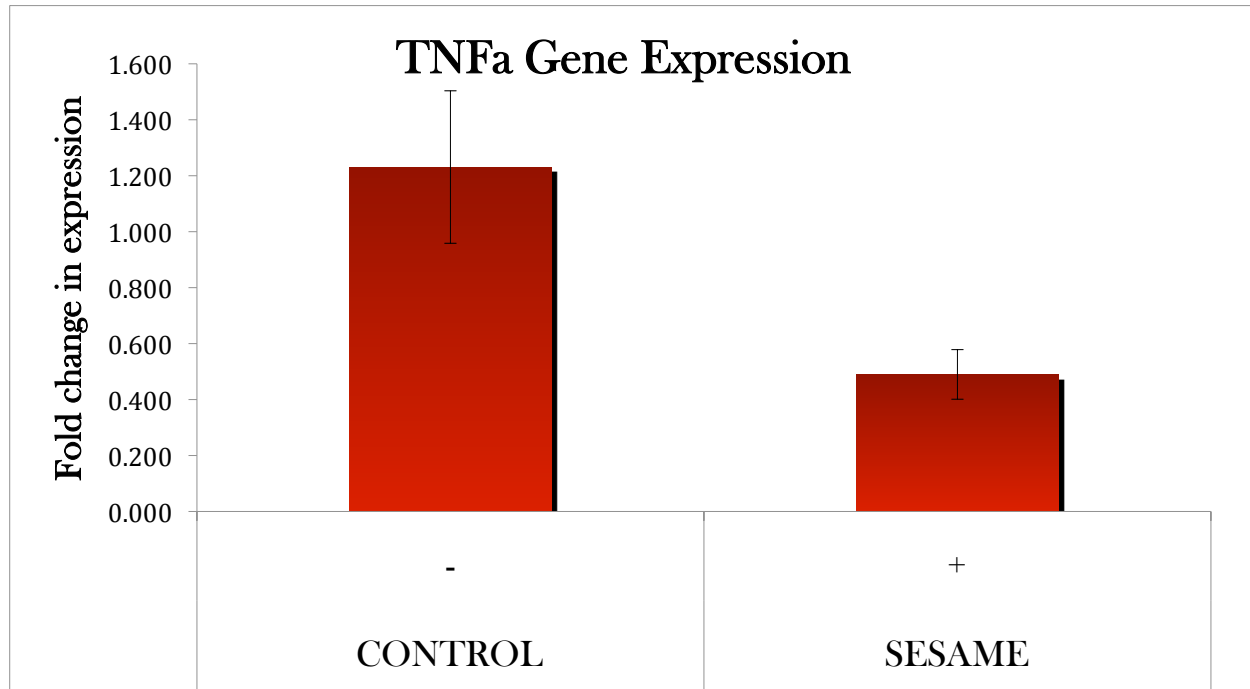


Figure 3: Fold change in expression of TNF α mRNA levels in the control group and sesame oil groups. The sesame oil fed mice had significantly ($p < 0.05$) decreased TNF α mRNA levels compared to the control group.

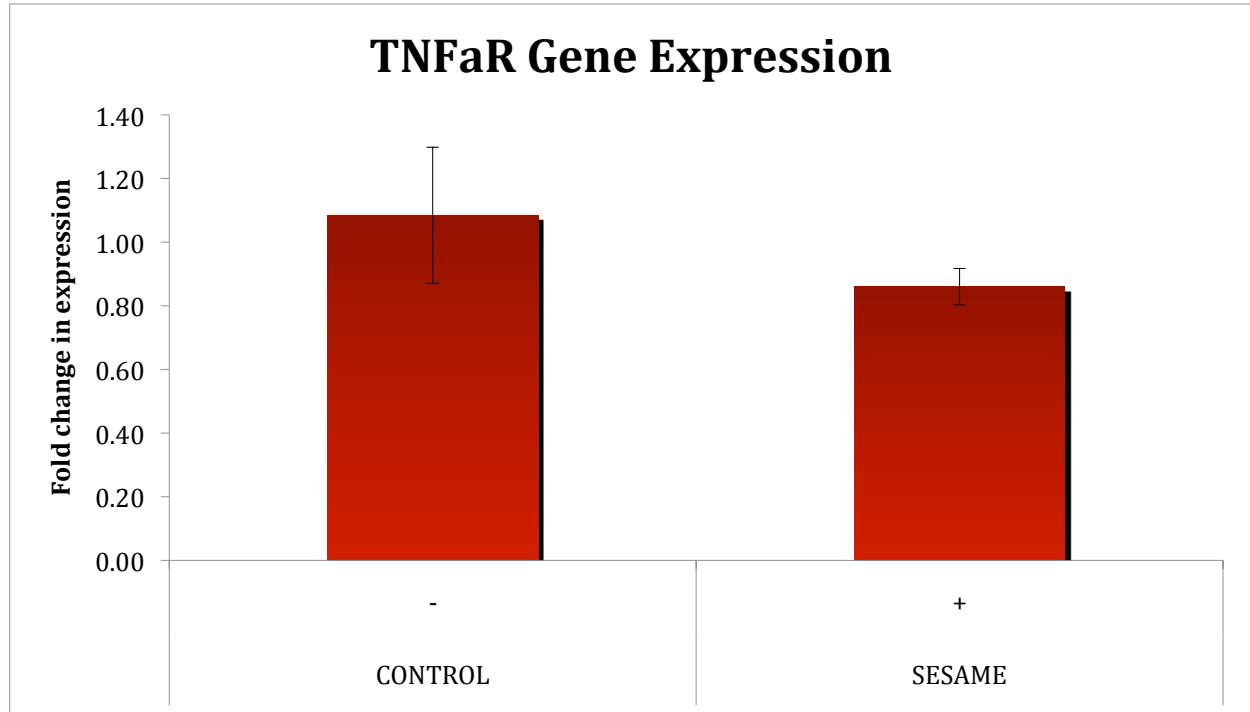


Figure 4: Fold change expression of TNF α R mRNA levels in the control and sesame oil groups. There was no statistical significance between the two groups.

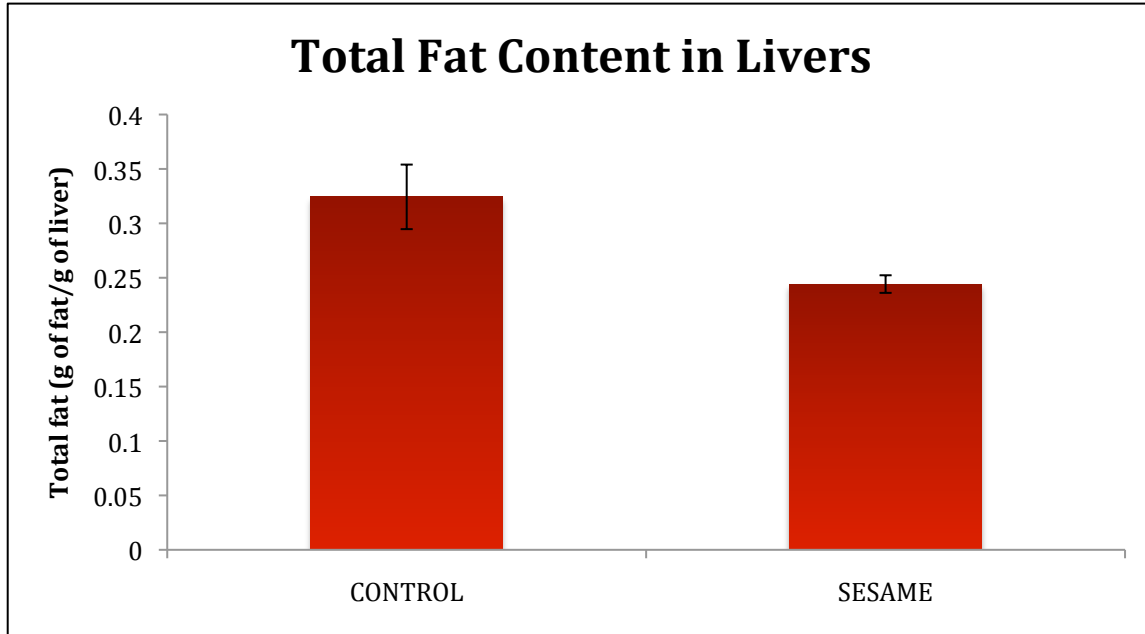


Figure 5: Total fat content of sesame oil and control fed mice. The sesame oil fed mice had significantly ($p<0.05$) lower amounts of total fat.

Sesame Diet	Diet	Liver
Saturated fat (%)	14	21
Monounsaturated fat (%)	39	44
Polyunsaturated fat (%)	41	40
Omega 6: Omega 3 ratio	135:1	19:1

Table 1: Fatty acid composition of diet and liver for mice fed the sesame oil diet.

<u>Control diet</u>	Diet	Liver
Saturated fat (%)	64	27
Monounsaturated fat (%)	31	61
Polyunsaturated fat (%)	4	12
Omega 6: Omega 3 ratio	7:1	2:1

Table 2: Fatty acid composition of diet and liver for mice fed the control diet.

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